Altered Sirtuin 7 Expression is Associated with Early Stage Breast Cancer

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ABSTRACT

BACKGROUND: To evaluate sirtuin-7 (SirT7) mRNA expression status in breast cancer patients with different metastatic stages and survey SirT7 mRNA expression status in eight different types of cancer.

METHODS: The expression of SirT7 in the commercially available TissueScan qPCR Breast Cancer Disease cDNA arrays containing 16 normal, 23 Stage I, 36 IIA, 22 IIB, 8 IIIA, 23 IIIA, 6 IIIB, 13 IIIC, and 5 IV were evaluated by quantitative real-time polymerase chain reaction (qRT-PCR) assay. Similar analysis was performed in TissueScan qPCR Cancer Survey cDNA array, which includes breast, colon, kidney, liver, lung, ovarian, prostate, and thyroid specimens.

RESULTS: The mRNA expression levels of SirT7 were significantly higher in breast cancer samples compared to normal breast specimens ($P < 0.001$). Stratification of patients into groups according to metastatic stages indicated statistically significantly higher levels of SirT7 mRNA in CS-I, CS-II, and CS-III when compared to normal breast tissue ($P < 0.05$). Notably, SirT7 mRNA levels were higher in CS-I, CS-IIA, CS-IIB, and CS-IIIA ($P < 0.05$). Additionally, there were significantly lower SirT7 mRNA levels in thyroid carcinoma when compared to their corresponding normal tissue ($P < 0.05$).

CONCLUSIONS: Our results indicate an increase in the mRNA expression level of SirT7 in breast cancer, particularly in CS-I, CS-IIA, CS-IIB, and CS-IIIA. The relationship of altered SirT7 with breast cancer progression and patient survival should be prospectively explored in future studies.

KEYWORDS: sirtuin-7, breast cancer, metastasis, cDNA arrays

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Introduction

According to a longstanding hypothesis, breast cancer seems to clinically and pathologically manifest as a sequence of defined stages according to the extent of disease starting with carcinoma in situ, progressing into invasive lesion and culminating in metastatic disease. However, individual prognosis varies significantly within each subgroup, indicating the heterogeneity of the current tumor stages. Metastasis by tumor cells represents the most dangerous attribute of cancer. When breast carcinomas remain confined to breast tissue, cure rates exceed 90%. As cells metastasize, however, long-term survival decreases depending upon the extent and the sites of colonization.

Cancer cells sequentially acquire hallmarks of malignancy as a result of genetic alterations, which drive the progressive transformation of normal cells into highly malignant derivatives. Several studies have suggested that the potential for distant metastasis and overall survival probability may be attributable to biological characteristics of the primary tumor, reflected by a specific gene expression signature.[1–4] Molecularly characterized lesions that play a causative role in tumorigenesis constitute more than 1% of the human genome.

Cancer is a disease of aging, as the incidence of most cancers increases with age following an accumulation of mutations. The Sir2 histone deacetylase gene family, consisting of seven mammalian sirtuins (SirT1–7), comprises the genes implicated in cellular and organismal aging. SirT1 has been extensively studied compared to other sirtuins. Several physiological SirT1 substrates have been identified, including p53, TAF_I68, MyoD, FOXO3, PPAR, NF-κB, and HIV-TAT.[5] On the other hand, few studies have explored the role of SirT7 in diseases. Low levels of SirT7 are found in nonproliferating tissues, such as heart, brain, and skeletal muscle.[6] SirT7 interacts with RNA polymerase I (Pol I) and promotes active transcription of rRNA genes.[7] Overexpression of SirT7 increased RNA Pol I-mediated transcription, whereas knockdown of SirT7 or inhibition of its catalytic activity resulted in decreased association of Pol I with rDNA and reduced Pol I transcription. Depletion of SirT7 stopped cell proliferation and triggered apoptosis.[7] Transcription of rDNA by Pol I accounts for

up to 65% of total transcription in metabolically active mammalian cells.[8] Pol I transcription is highly coordinated with cellular metabolism and cell proliferation.[9] Nutrient starvation, growth factor deprivation, DNA damage, and other conditions that slow down cellular division decrease pre-rRNA synthesis,[8] whereas conditions that stimulate cell growth and proliferation increase pre-rRNA synthesis.[10] These data suggest that SirT7 is an anti-senescence protein, and its inhibition results in senescence and apoptosis. Indeed, SirT7 knockout mice age prematurely and have a decreased lifespan.[11]

Elevated SirT7 expression has been detected in breast and thyroid carcinoma cell lines and tissues.[12,13] However, the relationship between SirT7 expression and cancer progression is unclear. In this study, we measured SirT7 mRNA expression in breast cancer specimens with different stages. We also surveyed eight different types of cancer for SirT7 mRNA expression levels.

Materials and Methods

Taqman qRT-PCR and cDNA array analysis. Primers were designed based on theoretically optimal conditions, which included primer melting temperature, primer annealing temperature, GC content, cross homology, and primer secondary structures. All primers were purchased from Bio Basic Canada Inc. A specific labeled probe (5′ FAM, 3′ BHQ-1) for each primer pair was used to ensure specificity of polymerase chain reactions (PCRs). Sequences for all primer/probe combinations are as follows:

- SirT7—forward primer 5′-GTGGACACTGCTTCAGAAAG-3′; reverse primer: 5′-CACAGTTCTGAGACACCACA-3′; probe: 5′-GTCTGCATGAGCAGAAGCTG-3′;
- Ubiquitin—forward primer: 5′-ACTACAACATCCAGAAAGAGTCCA-3′; reverse primer: 5′-CCAGTCAGGGTCTTCACGAAG-3′; probe: 5′-CCCACCTCTGAGACGGAGCACCAG-3′;
- RPL-13—forward primer: 5′-AACAAGTTGAAGTACCTGGCTTTC-3′; reverse primer: 5′-TGGTTTTGTGGGGCAGCATA-3′; probe: 5′-CGCAAGCGGATGAACACCAACCCT-3′;
- β-Actin—forward primer: 5′-TGACTGACTACCTCATGAAGATCC-3′; reverse primer: 5′-CCATCTCTTGCTCGAAGTCCAG-3′; probe: 5′-CGGCTACAGCTTCACCACCACGGC-3′.

A reaction volume of 20 μL was used for Taqman quantitative real-time (qRT)-PCR. The probe reaction assay consisted of 100 mM KCl, 20 mM Tris, pH 9.2, 5 mM $MgSO_4$, 0.02% Triton X-100, 0.2 mM dNTP, 200 mM Betaine, 5% DMSO, 1.25 IU hot-start Taq polymerase, 0.2 μM sense/anti-sense primers, and 2 μL cDNA. qRT-PCR reactions were run on a Cepheid Smartcycler. The reaction protocol consisted of one activation cycle of 50°C for two minutes followed by 95°C for 15 seconds. After this step, 40 cycles of denaturation at 95°C for 15 seconds and annealing/extension at 60°C for two minutes were performed. SirT7 cDNA was used to confirm qRT-PCR specificity reactions. SirT7 cDNA was purchased from OriGene. The amplified PCR products were sequenced. All samples were normalized to the expression level of the housekeeping gene β-actin. Comparative ΔΔCt method was used for the calculations of the normalized relative mRNA expression, as described previously.[14] Multiple sets of analyses with different housekeeping genes (RPL-13 and ubiquitin) were consistent. The number of replicates and the composition of the samples varied depending on the particular experiment but were never less than triplicate. Average cycle threshold (Ct) values were used to determine sensitivity and specificity of the designed probes. The specificity of a primer pair was evaluated with other sirtuin cDNAs (SirT1–6). There was no amplification of other sirtuins that share significant homology with SirT7. The inter- and intra-assay coefficients of variability for the probe-based qRT-PCR assay established for SirT7 were less than 10%.

Human tissue samples. Taqman qRT-PCR was utilized to measure the expression of SirT7 in the commercially available TissueScan qPCR Breast Cancer Disease Panels I, II, and IV (BCRT101, BCRT102, BCRT104). These panels were purchased from OriGene Technologies. Breast cancer complementary DNA (cDNA) arrays included 16 normal, 23 Stage I, 36 IIA, 22 IIB, 23 IIIA, 6 IIIB, 13 IIIC, and 5 IV, whose clinical and pathological features are freely available at the following address: http://www.origene.com/qPCR/Tissue-qPCR-Arrays.aspx. Additionally, Taqman qRT-PCR was also utilized to measure SirT7 expression levels in TissueScan qPCR Cancer Survey cDNA array I (CSRT101, OriGene) containing 96 samples covering eight different cancer specimens of different histotypes (breast adenocarcinoma, colon adenocarcinoma, kidney carcinoma, liver carcinoma, ovarian adenocarcinoma, thyroid carcinoma, lung carcinoma, and prostate adenocarcinoma). TissueScan qPCR Cancer Survey cDNA array I includes breast (N:3, SI:2, SII:2, SIII:3, SIV:2), colon (N:3, SI:1, SII:3, SIII:4, SIV:1), kidney (N:3, SI:3, SII:1, SIII:3, SIV:2), liver (N:3, SI:3, SII:3, SIII:1, SIV:2), lung (N:3, SI:4, SII:2, SIII:3, SIV:1), ovarian (N:3, SI:3, SII:1, SIII:4, SIV:1), prostate (N:3, hyperplasia: 5, prostitis: 2 SII:1, SIII:1), and thyroid (N:3, SI:3, SII:2, SIII:2, SIV:2) specimens.

Statistical analysis of cDNA array data. Statistical analysis was performed using SigmaStat software version 3.5. Analysis was carried out with Mann–Whitney rank sum test between normal and cancer specimens. Kruskal–Wallis one-way analysis of variance (ANOVA) on ranks was used to compare normal specimen and different stages of cancer, followed by Dunn's test for all pairwise comparisons and comparisons against the control group.

Results

TaqMan qRT-PCR confirms increased mRNA levels of SirT7 in breast cancer. To evaluate the expression of SirT7

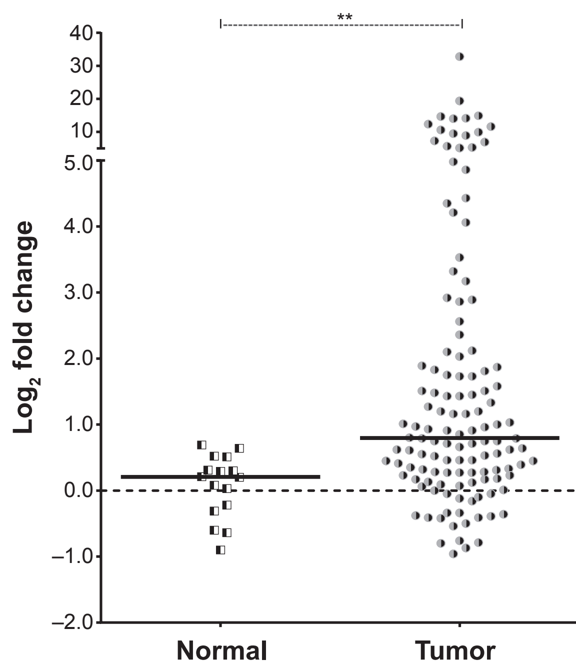

Figure 1. mRNA expression levels of SirT7 in normal and breast cancer specimens from Breast Cancer cDNA arrays (BCRT101, BCRT102 and BCRT104). The expression levels of SirT7 normalized to the expression levels of β-actin. Black bars represent the median $\log_2$ fold change value of SirT7. Asterisks indicate statistically significant differences (Mann-Whitney Rank Sum Test; $^{**}P < 0.001$; n = 16-normal, 128-breast adenocarcinoma specimens).

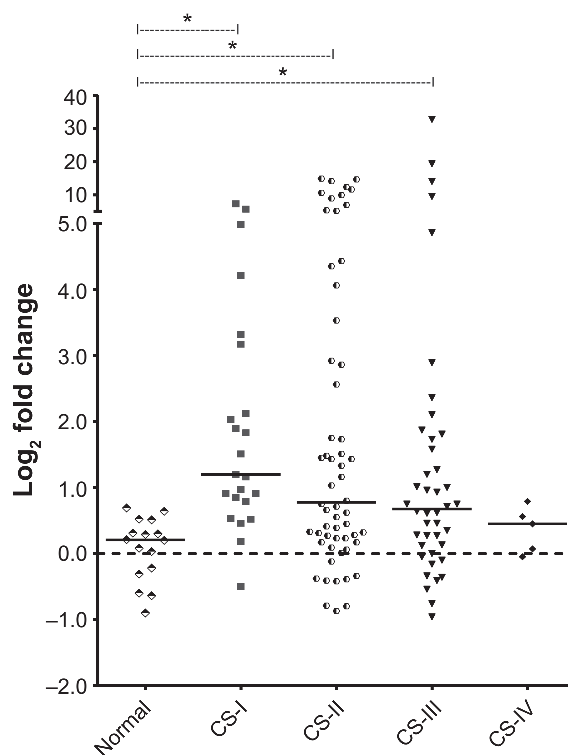

Figure 2. Relative mRNA expression levels of SirT7 in Breast Cancer cDNA arrays (BCRT101, BCRT102 and BCRT104) following stratification of patients into groups according to metastatic stage. The expression levels of SirT7 normalized to β-actin as a house keeping gene. Average normal ΔCt values were used for calculation of fold change differentials for each sample. Black bars represent the median $\log_2$ fold change value of SirT7; n = 16-normal, 23-Stage I, 58-II, 42-III, 5-IV. Asterisks indicate statistically significant differences (One Way ANOVA on Ranks; $^{*}P < 0.05$).

mRNA in breast cancer, we utilized the breast cancer cDNA arrays (BCRT101, BCRT102, and BCRT104) from OriGene Inc. The mean fold change differentials ($\log_2$-base scale) ± 95% CI of SirT7 in normal (n = 16) was 0.0694 ± 0.255. The mean fold change differentials ($\log_2$-base scale) ± 95% CI of SirT7 in tumor (n = 128) was 2.412 ± 0.803 (Fig. 1, $P < 0.001$).

Stratification of patients into groups according to metastatic stages confirms the increased mRNA levels of SirT7 in early stages of breast cancer. Clinical stage (CS) is an established indicator of breast cancer outcome. To determine SirT7 expression profiles of the premalignant, preinvasive, and invasive stages of breast cancer progression, a cDNA array platform containing 16 normal, 23 CS-I, 36 -IIA, 22 -IIB, 23 -IIIA, 6 -IIIB, 13 -IIIC, and 5 -IV was used. The data revealed statistically significantly higher levels of SirT7 mRNA (mean fold change differentials ± 95% CI) of SirT7 in CS-I (2.000 ± 0.837), CS-II (2.705 ± 1.109), and CS-III (2.477 ± 1.915) when compared to normal breast tissue (0.0694 ± 0.255; Fig. 2; $P < 0.05$). There was no statistical differences between CS-IV (0.364 ± 0.432) and normal tissues (Fig. 2). Further stratifications of metastatic stages into CS-IIA (2.541 ± 1.219), CS-IIB (2.973 ± 2.283), CS-IIIA (4.141 ± 3.440), CS-IIIB (0.520 ± 1.053), and CS-IIIC (0.435 ± 0.567) showed statistical significance only between CS-I, CSIIA, CS-IIB, and CS-IIIA and normal breast tissues (Fig. 3; $P < 0.05$).

Survey of the SirT7 mRNA expression in multiple tumor types. To examine whether increased SirT7 mRNA is a common event occurring in different cancer types, we used commercially available Cancer Survey cDNA array (CSRT101) containing eight different types of cancers with their corresponding normal tissues (breast, colon, kidney, liver, ovary, thyroid, lung, and prostate) to assess SirT7 expression status in tumor and normal tissues. Although the number of specimens included in the TissueScan qPCR Cancer Survey cDNA array was very small, there were significantly lower SirT7 levels in thyroid carcinoma when compared to their corresponding normal tissue ($P < 0.05$; Fig. 4). There were also higher SirT7 mRNA levels in the liver and ovary cancers but the "statistical analyses" were not significant, which could be due to small sample sizes.

Discussion

SirT7 apparently plays contradictory roles in cancer. Several studies suggest a role of SirT7 in helping to promote cell survival under stress conditions, which could be directly involved in tumorigenesis by inhibition of senescence and allowing unchecked cell division, while others suggest that SirT7

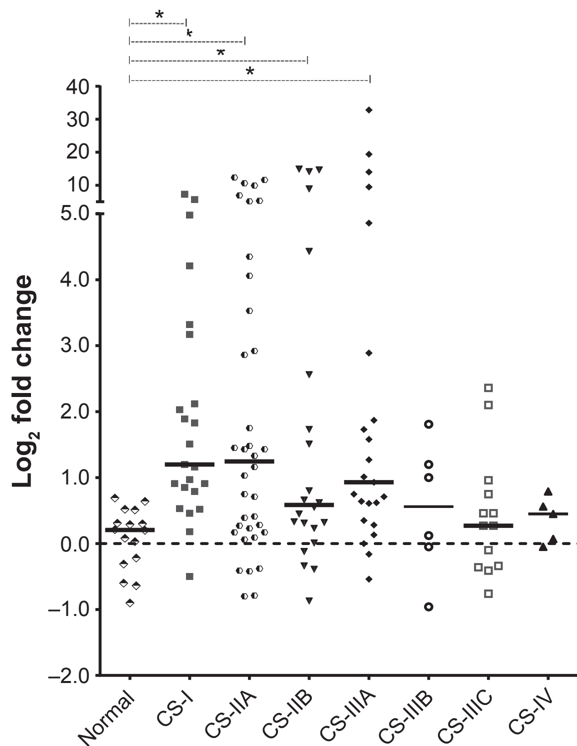

Figure 3. TaqMan qRT-PCR measurements of relative mRNA expression levels for SirT7 run on Breast Cancer cDNA arrays (BCRT101, BCRT102 and BCRT104) containing normal and cancer specimens with different metastatic stages. For each sample, the expression levels of SirT7 normalized to housekeeping gene (β-actin). Average normal ΔCt values were used for calculation of fold change differentials for each sample. Black bars represent the median $\log_2$ fold change value of SirT7. Asterisks indicate statistically significant differences (One Way ANOVA on Ranks; $^*P < 0.05$).

protects DNA (from damage and oxidative stress), maintains genomic stability, and limits replicative life span. SirT7 is widely expressed in proliferating mouse tissues, such as liver, testes, and spleen,[6,7] while low levels of SirT7 are found in nonproliferating tissues such as heart, brain, and skeletal muscle.[6] SirT7 levels decrease during aging in mouse hematopoietic stem cells[15] and during replicative senescence in normal human lung firbroblasts,[16] while its levels increase during passage of human mammary epithelial cells.[12]

SirT7 was found to be overexpressed in human thyroid papillary carcinoma compared to normal thyroid cells[13,17] and in breast cancers, with a significantly higher expression in node-positive than in node-negative breast cancers.[12] Recently, SirT7 expression was also found to be upregulated in a large cohort of human hepatocellular carcinoma patients.[18] SirT7 knockdown caused a significant increase of liver cancer cells to remain in the G1/S phase and to suppress growth. Upregulation of miR-125a-5p and miR-125b in human hepatocellular carcinoma cells suppressed SirT7 and cyclin D1 expression and induced p21(WAF1/Cip1)-dependent G1 cell cycle arrest. A regulatory loop was proposed, by which SirT7 inhibits transcriptional activation of p21(WAF1/Cip1).[18] In addition, SirT7-deficient hepatocellular carcinoma xenograft tissues also exhibit reduced tumor size[18] and inhibition of SirT7 expression in human U20S cells induced apoptosis,[7] further illustrating the prosurvival function of SirT7 in cancer cells. Since SirT7 regulates rRNA transcription, it is likely essential for cell survival and proliferation in both primary and transformed cells. It is unknown whether SirT7 elevation is a cause or consequence of cells bypassing senescence and inducing malignancy, because increased RNA Pol-I transcription of rRNA is required for tumor proliferation. Overall, these findings suggest that SirT7 is required for cell survival in the presence of xenotoxic stress as well as for normal proliferation.

On the other hand, SirT7 was shown to be involved in multiple pathways involved in ribosome biogenesis, and downregulation may contribute to its antitumor effect, partly through the inhibition of protein synthesis.[19] Our previous study demonstrated that inhibition of SirT7 expression in human breast MCF7 cell line induced senescence-associated β-galactosidase activity, reduced cell proliferation rate, inhibited caspase-8, induced drug resistance, and increased cell migration, suggesting that this gene may play an active role in regulating cancer cell response to stress. Additionally, inhibition of SirT7 in MCF7 cells resulted in inhibition of p53 and p21(WAF1/Cip1), and arrested cells at the G0/G1 phase.[20] This contradicts the proposed regulatory loop, whereby SirT7 inhibits transcriptional activation of p21(WAF1/Cip1).[18] We proposed SirT7 inhibition as a mechanism by which cells escape the toxic effect of chemotherapeutics by inducing a stress-induced premature senescence (SIPS)-like phenotype. Interestingly, a recent study in which Sir2 was overexpressed in drosophila flies resulted in the promotion of caspase-dependent but p53-independent apoptosis.[21] Furthermore, loss-of *Sir2* function prevented apoptosis induced by UV irradiation.[21] Our data are also consistent with the report by the Bober group[22] showing an antiproliferative effect of SirT7 in mouse fibroblasts, and SirT7 expression inversely correlated with the tumorigenic potential of several murine cell lines. Overexpression of SirT7 in mouse embryonic fibroblasts increased p53, pRB expression and induced c-Myc as well.[22] Additionally, SirT7 knockout in mouse embryonic fibroblasts showed higher viability than control wild-type mouse embryonic fibroblasts.[22] Further studies are required to elucidate this pathway. Thus, SirT7 apparently is bifunctional: operating as both a tumor suppressor and an oncogenic factor, although its role as a positive or negative factor might differ among organs and cell lineages.

Breast cancer is the most common malignancy among American women. Due to increased screening, the majority of patients present with early stage breast cancer.[23] Invasive breast cancer tumor stages range from CS-I to CS-IV, with CS-I having the best prognosis and CS-IV having the poorest. Here we carried out SirT7 gene expression profiling of breast cancer tissues, stages of the disease from early lesion (CS-I) to progressive stages (CS-IIB and IIIA–C), and identified

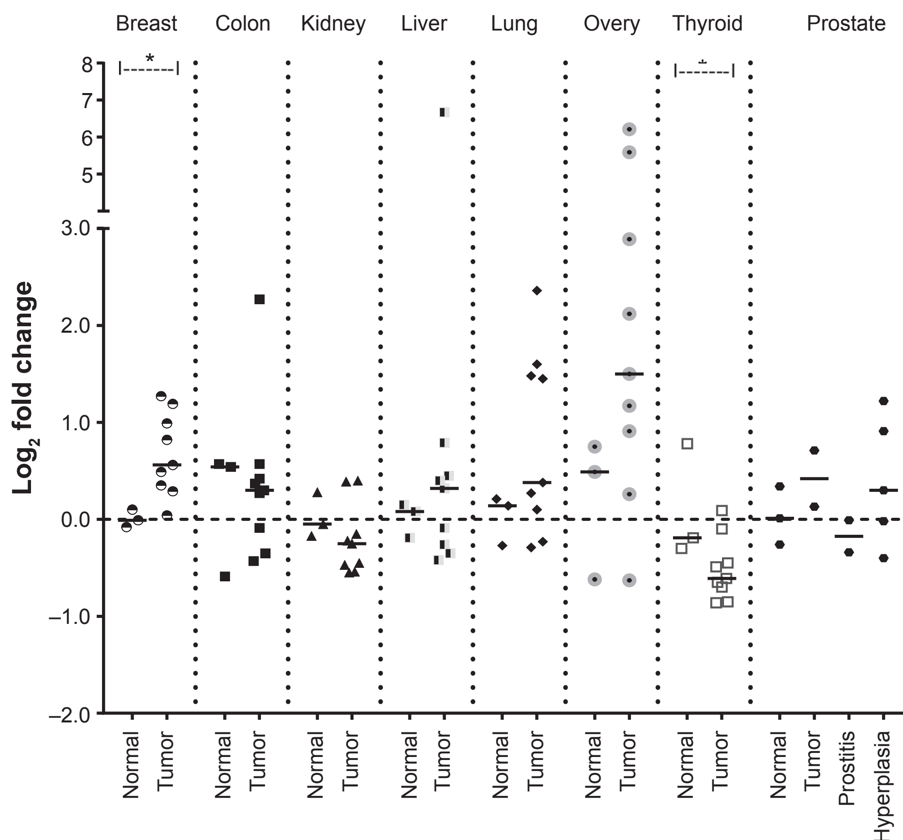

Figure 4. TaqMan qRT-PCR measurements of relative mRNA expression levels of SirT7 run on Cancer Survey cDNA arrays (CSRT101) containing 8 different types of cancers with their corresponding normal tissues (breast, colon, kidney, liver, ovary, thyroid, lung and prostate). The expression levels of SirT7 normalized to expression levels of β-actin housekeeping gene. Average normal ΔCt values were used for calculation of fold change differentials for each sample. Black bars represent the median $\log_2$ fold change value of SirT7. Asterisks indicate statistically significant differences (Mann-Whitney Rank Sum Test; $^*P < 0.05$).

distinct SirT7 expression signatures. It was found to be significantly upregulated in early stages of breast cancer. This suggests that overexpression of SirT7 protein is important at the earliest stages of breast oncogenesis and may continue to have a crucial role throughout the development of malignancy. Thus, overexpression of SirT7 is a feature of early stage breast cancer and may play a key role in mediating mitogenic responses to chemotherapy. On the other hand, there was no increase in SirT7 expression in breast cancer specimens in late stages (CS-IIIB to CS-IV). This could be a result of a smaller sample size. It would be of interest to examine SirT7 expression levels in patients undergoing chemotherapy, as the specimens examined were from nontreated patients. Unfortunately, the small number of samples included in the Cancer Survey cDNA panel limited the statistical analysis. However, there were significantly lower SirT7 mRNA levels in kidney and thyroid carcinomas when compared to the corresponding normal tissue.

The role of SirT7 in cancer remains a subject of open discussion. It may play critical roles in the maintenance of homeostasis and cellular metabolism in both normal and cancer cells. Our findings suggest the oncogenic potential of SirT7 is in early stages of breast cancers. Tumor cells may require higher levels of SirT7 to maintain rapid proliferation. Although the detailed mechanisms underlying these functions have not yet been elucidated, the identification of SirT7 substrates will improve our understanding of the functions and mechanisms of SirT7. There is growing evidence indicating that this research area should be prioritized to examine whether modulation of SirT7 could have implications for cancer treatment. However, the use of SirT7 inhibitors as potential cancer therapeutic agents might be impeded by an essential role of SirT7 in primary cells.

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Author Contributions

Conceived and designed the experiments: AA, MA. Analyzed the data: AA, HB, AS. Wrote the first draft of the manuscript: AA. Contributed to the writing of the manuscript: AN, AB. Agree with manuscript results and conclusions: AA, AB, HB, AN. Jointly developed the structure and arguments for the paper: AA, AS. Made critical revisions and approved final version: AN. All authors reviewed and approved of the final manuscript.

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